Regulation of glycogen metabolism in yeast and bacteria

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Abstract
Microorganisms have the capacity to utilize a variety of nutrients and adapt to continuously changing environmental conditions. Many microorganisms, including yeast and bacteria, accumulate carbon and energy reserves to cope with the starvation conditions temporarily present in the environment. Glycogen biosynthesis is a main strategy for such metabolic storage, and a variety of sensing and signaling mechanisms have evolved in evolutionarily distant species to ensure the production of this homopolysaccharide. At the most fundamental level, the processes of glycogen synthesis and degradation in yeast and bacteria share certain broad similarities. However, the regulation of these processes is sometimes quite distinct, indicating that they have evolved separately to respond optimally to the habitat conditions of each species. This review aims to highlight the mechanisms, both at the transcriptional and at the post-transcriptional level, that regulate glycogen metabolism. In the yeast system, we focus particularly on the various signaling pathways that control the activity of the enzymes of glycogen storage. We also discuss our recent understanding of the important role played by the vacuole in glycogen metabolism. In the case of bacterial glycogen, special emphasis is placed on aspects related to the genetic regulation of glycogen metabolism and its connection with other biological processes.

Introduction
Many microorganisms accumulate carbon and energy reserves to cope with the starvation conditions temporarily present in the environment. The biosynthesis of glycogen is a main strategy for such metabolic storage. Glycogen is a major intracellular reserve polymer consisting of α-1,4-linked glucose subunits with α-1,6-linked glucose at the branching points. In bacteria, the average length of the chains is 8–12 glucose units, and the molecular size of glycogen has been estimated to be about $10^7$–$10^8$ Da. The structure of yeast glycogen is similar to that of other glycogens, with a chain length of 11–12 glucose residues (Northcote, 1953) and a particle diameter of around 20 nm (Mundkur, 1960). In yeast, glycogen is formed upon limitation of carbon, nitrogen, phosphorous or sulfur (Lillie & Pringle, 1980), whereas in bacteria, this polyglucan accumulates under conditions of limiting growth when an excess of carbon source is available and other nutrients are deficient (Lillie & Pringle, 1980; Eydallin et al., 2007b; Montero et al., 2009). An exception to this, where glycogen accumulates to optimal levels during the exponential growth phase, has been observed with cultures of Streptococcus mitis (Gibbons & Kapsimalis, 1963) and Rhodopseudomonas capsulata (Eidels & Preiss, 1970). In yeast, significant quantities of glycogen are synthesized and degraded as diploid yeast cells undergo the sporulation process (Colonna & Magee, 1978).

One outstanding advantage in using glycogen as a reserve compound is that this macromolecule has little effect on the internal osmotic pressure of the cell. In yeast, the importance of glycogen reserves in survival during long-term
nutrient deprivation has been demonstrated clearly (Sillje et al., 1999). Furthermore, yeast cells that can accumulate glycogen stores have a growth advantage over cells that cannot, suggesting that glycogen makes a contribution toward overall fitness (Anderson & Tatchell, 2001). The exact role of this polyglucan in bacteria is not as clear-cut as in animal and yeast cells, but several studies have linked bacterial glycogen metabolism to environmental survival, symbiotic performance and colonization and virulence (Bonafonte et al., 2000; Marroqui et al., 2001; Henrissat et al., 2002; Lepek et al., 2002; Chang et al., 2004; McMeechan et al., 2005; Jones et al., 2008; Sambou et al., 2008; Bourassa & Camilli, 2009). Experiments carried out early in the 1960s suggested that bacteria need glycogen to provide energy for maintenance under nongrowing conditions.

Several excellent reviews on yeast storage carbohydrate metabolism have been published. The most recent dates from 2001 (Francois & Parrou, 2001) and, in the 8 or so years since its publication, some significant advances in our understanding of glycogen storage have been made. For a comprehensive review of the older literature, the reader is referred to Francois & Parrou (2001). Ballicora et al. (2003) and Preiss (2009) have published reviews on aspects relating to the allosteric properties and structure–function relationships of enzymes directly involved in bacterial glycogen metabolism, to which readers are referred for further information. Our aim here is to focus on selected areas where the greatest increase in knowledge has occurred.

The regulation of glycogen metabolism in yeast

The enzymes of yeast glycogen storage and utilization

The synthesis of glycogen requires the activities of glycogenin, a self-glucosylating initiator protein (Farkas et al., 1991; Cheng et al., 1995), glycogen synthase (Farkas et al., 1991), which catalyzes bulk synthesis, and the branching enzyme, which introduces the α-1,6-branch points characteristic of glycogen. Degradation occurs via the concerted action of glycogen phosphorylase, which releases glucose as glucose-1-phosphate from linear α-1,4-linked glucose chains, and the debranching enzyme, which eliminates the α-1,6-branch points. Alternatively, glycogen can be hydrolyzed in the vacuole by a glucoamylase activity, generating free glucose. See text for details.

UDPG pyrophosphorylase

UDPG is synthesized from UTP and glucose-1-phosphate. This reaction is catalyzed by the enzyme UDPG pyrophosphorylase (EC 2.7.7.9), which is encoded by a single gene, UGP1 (Daran et al., 1995). The UGP1 gene is essential due to the pivotal role played by UDPG in yeast metabolism. Protein N-glycosylation, utilization of galactose as a carbon source, production of trehalose and synthesis of cell wall β-glucan all require UDPG (Daran et al., 1997) (Fig. 2).

During the logarithmic growth of cells, there is a large demand for UDPG for cell wall β-glucan synthesis to be
maintained. This substantial UDPG demand arises because each new daughter cell clearly requires its own cell wall. However, as cells enter the stationary phase of growth and cell division slows, glycogen synthesis is initiated. What mechanisms regulate the channeling of UDPG between these various processes? Recent work from Rutter’s group has demonstrated convincingly the existence of signaling pathways that coordinate channeling of UDPG into either glucan synthesis or the production of cell wall material in response to both nutritional status and cell integrity signaling (Grose et al., 2007; Smith & Rutter, 2007).

Rutter et al. (2002) identified two yeast kinases, Psk1p and Psk2p, as important for growth in a galactose-containing medium at elevated temperature. These kinases contain a conventional serine/threonine kinase domain associated with two PAS domains, and are part of a family of enzymes conserved from yeast to humans (Rutter et al., 2001, 2002; Amezcua et al., 2002). The PAS domains are conserved signaling modules that act as sensors for a wide variety of stimuli, including oxygen, light and small-molecule ligands (Taylor & Zhulin, 1999). Recombinant Psk2p was shown to phosphorylate Ugp1p. This phosphorylation did not alter the activity of Ugp1p. However, failure to phosphorylate Ugp1p in vivo led to inadequate cell wall β-glucan synthesis, a weakened cell wall and increased glycogen accumulation (Smith & Rutter, 2007). Therefore, phosphorylation altered the fate of the UDPG synthesized by the enzyme. This channeling of UDPG toward specific fates appeared to be achieved through the control of Ugp1p localization. Phosphorylated Ugp1p localized to the plasma membrane, whereas the dephosphorylated enzyme was found in the cytoplasm (Smith & Rutter, 2007). The synthesis of cell wall β-glucans occurs at the plasma membrane, while glycogen synthesis is a cytoplasmic process (Cid et al., 1995; Montijn et al., 1999; Huh et al., 2003; W.A. Wilson, unpublished data). Thus, the phosphorylated and dephosphorylated forms of Ugp1p govern the fate of UDPG by virtue of their localization to the site of β-glucan synthesis or glycogen synthesis, respectively.

Rutter’s group has established that the yeast PAS kinases are regulated in response to both cell integrity stress and nutrient conditions (Grose et al., 2007). The regulation is quite complex and, for example, it appears that cell wall stress activates both Psk1p and Psk2p, whereas Psk1p alone responds to nonfermentable carbon sources (Grose et al., 2007).

Glycogenin

Eukaryotic glycogen synthases cannot initiate the synthesis of a glycogen particle de novo. Rather, they function to elongate a pre-existing oligosaccharide primer, which is attached to a protein referred to as glycogenin (EC 2.4.1.186). There are two isoforms of glycogenin in yeast, encoded by GLG1 and GLG2 (Cheng et al., 1995). The protein products are 67 and 43 kDa, respectively. Despite this significant size difference, the two proteins appear to be functionally redundant and the deletion of either GLG1 or GLG2 has no effect on glycogen storage, whereas a glg1 glg2 double mutant is glycogen-deficient (Cheng et al., 1995).

Although the initiation steps in glycogen storage might appear to be attractive candidates for regulation, there is no good evidence for the post-translational control of glycogenin activity in yeast. The glycogenins are, however, both regulated at the level of transcription, induction of expression being observed at the diauxic shift (Cheng et al., 1995; DeRisi et al., 1997). Glycogenin serves as an initiator of glycogen synthesis, catalyzing two distinct reactions, which are referred to as the self-glucosylation and elongation reactions, respectively (Mu et al., 1996; Roach & Skurat, 1997). In the self-glucosylation reaction, glucose is transferred from UDPG to a tyrosine residue within glycogenin, forming an unusual glucose 1-O-tyrosyl linkage (Mu et al., 1996; Roach & Skurat, 1997). In the elongation reaction, additional glucose moieties are added in α-1,4-glycosidic linkage, forming a chain of approximately 8–10 glucose residues. Again, the glucose donor is UDPG. The oligosaccharide bound to glycogenin then serves as the
substrate for glycogen synthase (Roach & Skurat, 1997). The crystal structure of rabbit muscle glycogenin was resolved in 2002 (Gibbons et al., 2002). However, precisely how glycogenin catalyzes the distinct reactions of self-glucosylation (formation of a glucose–tyrosine linkage) and elongation (formation of α,1,4-glycosidic linkages) remains unclear.

Glycogen accumulation can be readily assessed in yeast using the simple procedure of exposing colonies of cells growing on the surface of an agar plate to iodine vapor (Chester, 1968). Yeast cells stain brown in proportion to the amount of glycogen that they contain. A wild-type (WT) yeast strain stains brown while a double mutant yeast strain stains yellow, indicating that glycogen is not being produced (see, e.g. Cheng et al., 1995; Hurley et al., 2005; Torija et al., 2005). However, iodine staining of a population of ggl1 ggl2 double mutant cells revealed that ~2–3% accumulated glycogen (Torija et al., 2005). This glycogen-storage phenotype was reversible and dependent on glycogen synthase. Furthermore, it was considerably enhanced by expression of an activated form of glycogen synthase or by deletion of the TPS1 gene that encodes trehalose-6-phosphate synthase (Guillou et al., 2004; Torija et al., 2005). The ability to store glycogen was not stably transmitted between mother and daughter cells. The synthesis of glycogen in the absence of glycogenin therefore appeared to be stochastic in nature. Presumably, there must be some gratuitous primer molecule containing an oligosaccharide (a glycoprotein perhaps), to which glycogen synthase added glucose units. This would be a rare event, possibly enhanced by the expression of an activated glycogen synthase.

**Glycogen synthase**

Just as there are two isoforms of glycogenin, yeast cells also contain two isoforms of glycogen synthase (EC 2.4.1.11), encoded by the GSY1 and GSY2 genes, respectively (Farkas et al., 1991). The protein products are 80% identical. The deletion of the GSY1 gene results in an approximately 90% decrease in glycogen synthase activity during late logarithmic growth (Farkas et al., 1991). The glycogen content of gsy2 mutant cells is also much reduced relative to WT (Farkas et al., 1991). In view of these observations, Gsy2p has been considered the major form of glycogen synthase and most characterization efforts have focused on this, rather than the Gsy1p isoform.

Transcription of GSY2 is strongly induced toward the end of the logarithmic growth phase and this induction requires the cyclic AMP (cAMP)-dependent protein kinase pathway (PKA; discussed in more detail below) (Farkas et al., 1991; Hardy et al., 1994). Post-translational control is also extremely important in the regulation of Gsy2p and the enzyme can be inhibited by phosphorylation (Huang & Cabib, 1974; Francois & Hers, 1988). The inhibition by phosphorylation is overcome by the presence of the allosteric activator glucose-6-phosphate (glucose-6-P) (Huang & Cabib, 1974; Francois & Hers, 1988; Hardy & Roach, 1993; Pederson et al., 2000). Glucose-6-P has little effect on the activity of the dephosphorylated enzyme. The activity measured in the presence of a saturating amount of glucose-6-P is therefore referred to as the total activity because it represents the contribution of all the glycogen synthase molecules present regardless of the phosphorylation state. The ability of glucose-6-P to restore activity to phosphorylated and inactivated glycogen synthase forms the basis of the −/+glucose-6-P activity ratio that is used as an index of activation state. A high activity ratio indicates that glycogen synthase is predominantly in the active, dephosphorylated form while a low ratio shows that phosphorylated, inactive glycogen synthase is present (Huang & Cabib, 1974; Francois & Hers, 1988; Hardy & Roach, 1993; Pederson et al., 2000). The control of glycogen synthase by phosphorylation and glucose-6-P is discussed in detail below.

**Branching enzyme**

As mentioned above, glycogen is a branched polymer. The branching is important for glycogen function because both the synthesis and the degradation of the polymer occur from the nonreducing ends of the α,1,4 chains (Roach et al., 2001). Branching increases the number of ends and hence the rate at which glycogen can be both degraded and resynthesized. Additionally, the water solubility of glucose polymers increases with the extent of branching, and less-branched polymers, such as amylose, are less soluble than glycogen (Smith, 2001).

Glycogen synthase catalyzes the synthesis of only α,1,4 linkages and the α,1,6 branch points are introduced by a carbohydrate-remodeling activity known as the branching enzyme (EC 2.4.1.18), which is encoded by GLC3 (Rowen et al., 1992). Branching enzyme transfers a block of six or seven glucose residues from the end of a linear chain of glucose residues linked by α,1,4 bonds, and attaches this block via an α,1,6 linkage to a more interior glucose residue (Gunja et al., 1960; Manners, 1971). Deletion of GLC3 has been reported to result in considerably reduced carbohydrate storage (Rowen et al., 1992). As with glycogen synthase, transcription of GLC3 is induced at the approach to the stationary phase, concomitant with the onset of glycogen storage (Rowen et al., 1992; DeRisi et al., 1997). There is no evidence for post-translational control of Glc3p and a key regulator of glycogen branching appears to be the balance between the activity of glycogen synthase and that of the branching enzyme (Raben et al., 2001; Pederson et al., 2003; Wilson et al., 2004).
Glycogen phosphorylase, glucoamylase and the debranching enzyme

Degradation of glycogen in yeast can proceed via two different pathways. First, glycogen can be degraded by glycogen phosphorylase (EC 2.4.1.1; GPH1 gene product), which releases glucose in the form of glucose-1-phosphate from the nonreducing ends of α-1,4-linked chains (Hwang et al., 1989). Second, free glucose can be generated from glycogen via hydrolysis, which is catalyzed by a vacuolar glucoamylase (EC 3.2.1.3, encoded by the SGA1 gene) (Colonna & Magee, 1978; Yamashita & Fukui, 1985; Pugh et al., 1989). The role of Sga1p has been somewhat underappreciated in the control of glycogen utilization. This is likely due to its original description as a sporulation-specific enzyme, responsible for the mobilization of glycogen reserves in germinating yeast spores (Colonna & Magee, 1978; Yamashita & Fukui, 1985; Pugh et al., 1989). The role of Sga1p has been somewhat underappreciated in the control of glycogen utilization. This is likely due to its original description as a sporulation-specific enzyme, responsible for the mobilization of glycogen reserves in germinating yeast spores (Colonna & Magee, 1978; Yamashita & Fukui, 1985; Pugh et al., 1989). It is now understood that Sga1p plays a role in glycogen metabolism late in the growth period, even in haploid cells (Wang et al., 2001a). A discussion of our current understanding of the importance of this protein is presented below.

Expression of GPH1 is induced as cells approach the stationary phase, similar to many other genes involved in glycogen storage and/or utilization (Hwang et al., 1989). As with glycogen synthase, this increase in expression requires the activity of the PKA pathway (Sunnarborg et al., 2001). Yeast glycogen phosphorylase is activated by phosphorylation (Fosset et al., 1971; Lerch & Fischer, 1975; Francois & Hers, 1988). The metabolite glucose-6-P plays a key role in the control of yeast glycogen phosphorylase, this compound serving to facilitate dephosphorylation and inactivation of the enzyme (Lin et al., 1996). There have been some notable developments in our understanding of the regulation of glycogen phosphorylase in yeast since the field was last reviewed and these newer findings are discussed below.

Yeast glycogen phosphorylase is capable of removing glucose residues only from α-1,4-linked glucose chains. The enzyme is incapable of dealing with α-1,6 branch points and indeed stops two to three glucose residues away from them. A debranching enzyme (EC 2.4.1.25; encoded by GDB1) is required to handle the branch points (Teste et al., 2000). Gdb1p catalyzes two sequential reactions. First, the maltotriose (or maltose) unit is transferred from the branch point to the nonreducing end of an adjacent α-1,4-linked glucose chain. Second, the residual α-1,6-linked glucose residue is hydrolyzed. Phosphorylase activity then proceeds, degrading glycogen until the next branch point is reached (Lee et al., 1970; Tabata & Hizukuri, 1992; Teste et al., 2000). As would be predicted, deletion of GDB1 impairs the ability of yeast to mobilize glycogen (Teste et al., 2000). There is no evidence for post-translational control of branching enzyme activity in yeast. Similar to the other enzymes of glycogen metabolism, the expression of GDB1 is increased at the approach to the stationary phase in batch culture (Teste et al., 2000). The end product of glycogen catabolism via the concerted action of glycogen phosphorylase and the debranching enzyme is a mixture comprising mostly glucose-1-phosphate and also a small quantity of glucose.

Transcriptional control of glycogen storage and utilization

As indicated above, the expression of glycogen synthase, glycogen phosphorylase, the branching enzyme, the debranching enzyme and glycogenin is tightly regulated. The expression of the genes encoding each of these enzymes is induced as cells approach the stationary phase of growth in batch culture and the PKA pathway is central to this control. Mutations that activate PKA result in decreased glycogen storage, whereas mutations that downregulate the PKA pathway result in hyperaccumulation of glycogen (Tatchell et al., 1985; Toda et al., 1985, 1987; Cannon et al., 1986; Cannon & Tatchell, 1987; Levin et al., 1988; Tanaka et al., 1990). As described above, and discussed in detail by Francois & Parrou (2001), the PKA pathway controls transcription of the GSY1, GSY2, GLG1, GLG2, GPH1, GLC3 and GDB1 genes. In the last few years, there has been a significant improvement in our understanding of the transcriptional control of the GSY2 and GSY1 genes and this will be discussed below. Figure 3 provides a summary of our current understanding of the promoter regions of GSY2 and GSY1. The reader is referred to Francois & Parrou (2001) for a discussion of historical findings and a comprehensive listing of references.

Control of GSY2 expression by the PKA pathway

PKA-mediated transcriptional control of GSY2, and indeed probably all genes regulated by PKA, involves at least in part the transcription factors Msn2p and Msn4p (reviewed by Francois & Parrou, 2001 in relation to glycogen storage). These are largely redundant proteins that are key regulators of the general stress response in yeast (Martinezpastor et al., 1996). Deletion of either MSN2 or MSN4 yields a cell with no obvious phenotypic change from the WT (Estruch & Carlson, 1993). However, deletion of both MSN2 and MSN4 yields a cell that is hypersensitive to stress and unable to synthesize glycogen (Estruch & Carlson, 1993). Msn2p and Msn4p exert their function through binding to a short DNA motif, known as the STRE element, present in the promoter of around 200 or so stress-sensitive genes (Estruch, 2000; Causton et al., 2001).

Under basal conditions, Msn2p and Msn4p are maintained in the cytoplasm (Gorner et al., 1998). Stress
conditions allow translocation into the nucleus and subsequent activation of Msn2p/Msn4p-sensitive genes (Gorner et al., 2002). The nuclear translocation is controlled, at least in part, by the PKA pathway and is best characterized for Msn2p. A current model holds that Msn2p is directly phosphorylated by PKA under basal conditions, maintaining the protein in the cytoplasm (Gorner et al., 2002). The entry of Msn2p into the nucleus takes place subsequent to a reduction in Msn2p phosphorylation, which occurs either through downregulation of the PKA pathway or activation of specific protein phosphatases (Gorner et al., 2002; De Wever et al., 2005).

The promoter of the GSY2 gene contains two STRE elements (Ni & Laporte, 1995). Francois and colleagues demonstrated that the deletion of these two elements abolished the induction of GSY2 expression that is normally seen in response to nitrogen starvation, glucose starvation or heat stress (Parrou et al., 1999b). However, removal of the STRE elements did not block the increase in the transcription of GSY2 that occurred upon the approach to the stationary phase (although the magnitude of the induction was reduced around 20-fold) (Parrou et al., 1999a). Additionally, a yeast strain in which the GSY2 gene was replaced by a mutant form lacking STRE elements in the promoter was constructed. This yeast strain initiated glycogen synthesis during growth, ultimately accumulating levels similar to WT cells and did so over a similar time course (Enjalbert et al., 2004). The total glycogen synthase activity in the strain with the mutant promoter was less than that seen in WT cells, but, again, the glycogen synthase activity was found to increase during growth, with kinetics indistinguishable from those observed in the WT strain (Enjalbert et al., 2004). In addition, when the STRE elements were removed from the GSY2 promoter, GSY2 expression was still responsive to Msn2p/Msn4p (Enjalbert et al., 2004). The control of GSY2 expression by the PKA pathway was therefore shown to involve at least both STRE-dependent and -independent, and Msn2p/Msn4p-dependent and -independent inputs. One of the STRE-independent, Msn2p-independent pathways appeared to operate through the basic helix–loop–helix transcription factor Sok2p, which was originally identified as a suppressor of the growth defect that arises due to a lack of PKA activity (Ward & Garrett, 1994).
Control of GSY2 expression by the SNF1 pathway

As with many other genes involved in the metabolism of carbohydrates, GSY2 is known to be subject to glucose repression, that is, transcription is downregulated in the presence of adequate glucose (Hardy et al., 1994). Relief from glucose repression requires the activity of a protein kinase, Snf1p, and the deletion of SNF1 prevents the derepression of glucose-repressed genes (Hedbacker & Carlsson, 2008; Turcotte et al., 2010). A major target of Snf1p is the Mig1p transcriptional repressor (reviewed recently in Turcotte et al., 2010). Phosphorylation of Mig1p by Snf1p results in its inactivation, allowing the derepression of glucose-repressed genes. Transcription of GSY2 is somewhat reduced in snf1 mutants, consistent with a role for Snf1p in the regulation of GSY2 expression (Hardy et al., 1994). The GSY2 promoter contains putative consensus Mig1p-binding sites and deletion of the MIG1 gene in an snf1 mutant yeast strain restored the expression of GSY2 to WT levels (Enjalbert et al., 2004). However, this effect is unlikely to be direct because obliteration of the potential Mig1p-binding sites from the GSY2 promoter is reported not to impact Snf1p-mediated regulation of expression (Enjalbert et al., 2004). Cells in which the SNF1 gene is deleted have considerably reduced levels of glycogen relative to WT cells. This reduction in glycogen is more severe than can be accounted for by the modest reduction in GSY2 transcription observed. Indeed, as discussed below, Snf1p has multiple inputs into glycogen storage beyond its role in the transcriptional control of GSY2.

Control of GSY2 expression via the cyclin-dependent kinase Pho85p

The PHO85 gene encodes the catalytic subunit of a cyclin-dependent protein kinase (Toh-e et al., 1988). Cells in which the PHO85 gene is deleted grow slowly, show constitutive expression of secreted acid phosphatases, cannot sporulate when diploid, show a defect in growth on nonfermentable carbon sources and have an aberrant morphology including a very large vacuole (reviewed in Huang et al., 2007). In addition, pho85 mutants also overaccumulate glycogen (Huang et al., 1996b; Timblin et al., 1996). (Strain-to-strain variability and Pho85p: It is worth pointing out here that the deletion of PHO85 has different effects on glycogen accumulation in different genetic backgrounds. In particular, Goding and colleagues have shown that deletion of PHO85 in a W303a background does not result in hyperaccumulation of glycogen. The majority of studies discussed in this review article use the EG328-1A background, which is a derivative of S288c. Here, the deletion of PHO85 results in robust glycogen hyperaccumulation.) Pho85p has multiple inputs into glycogen storage, not all of which are conceptually coherent at first sight. The transcriptional inputs are discussed below and additional, post-translational inputs are covered in a later section. Consistent with the pleiotropic role of the Pho85p kinase catalytic subunit, 10 Pho85p cyclins (Pcls) have been identified in yeast (Measday et al., 1997). The hypothesis is that the Pcls confer specificity to the catalytic subunit Pho85p (Andrews & Measday, 1998; Huang et al., 1998). The best-characterized Pcl is arguably Pho80p, which targets Pho85p to the transcription factor Pho4p, thereby regulating gene expression in response to phosphate availability (Kaffman et al., 1994, 1998a; b; O’Neill et al., 1996; Komeili & O’Shea, 1999; Lee et al., 2007). Roles for several other Pcl proteins have also been defined (reviewed by Huang et al., 2007).

Transcription of GSY2 was found to be increased in pho85 mutant cells (Timblin & Bergman, 1997). This increase in expression was independent of growth phase and pho85 mutants showed increased GSY2 expression relative to WT cells both in the logarithmic phase and at the approach to stationary phase (Enjalbert et al., 2004). Deletion analysis of regions of the GSY2 promoter revealed that a 14 base pair G/C-rich region was apparently required to confer regulation of expression by Pho85p (Enjalbert et al., 2004). The responsible DNA-binding factor has yet to be identified (Enjalbert et al., 2004). Likewise, the cyclin or cyclins with which Pho85p partners to achieve transcriptional regulation of GSY2 remains to be defined. As discussed below, when additional inputs of Pho85p into glycogen storage are considered, the related proteins Pcl6p and Pcl7p are likely candidates (Wang et al., 2001b).

Regulation of GSY2 in response to oxygen

Binding sites for the Hap2p protein, a component of the Hap2/3/4/5 complex have been identified in the GSY2 promoter (Parrou et al., 1999a). Hap2/3/4/5 functions as a heme-activated regulator of transcription (Zitomer & Lowry, 1993; McNabb et al., 1995; Parrou et al., 1999a; Schuller, 2003a; McNabb & Pinto, 2005). Heme synthesis is absolutely dependent upon the presence of oxygen and, therefore, the abundance of heme can serve as an indicator of oxygen status. The Hap2/3/4/5 complex is thus an activator of transcription under aerobic conditions. The presence of the Hap2p-binding sites in the GSY2 promoter indicated that the gene might be activated in response to oxygen availability. Deletion of a putative Hap2p-binding site did indeed reduce the maximal expression of GSY2 observed at the diauxic shift, albeit by only around 40% (Parrou et al., 1999a). This might seem modest but, given certain features of the control of GSY1 expression discussed below, the potential impact of oxygen on the control of GSY2 expression may be worthy of further consideration.
The roles of TOR in regulation of GSY2 transcription

Treatment of yeast cells with rapamycin evoked many of the same responses as does nutrient starvation, including glycogen accumulation (reviewed in De Virgilio & Loewith, 2006). This gave clear indication that the signaling pathways mediated by the TOR kinases had a role to play in the control of glycogen storage. The TOR kinases are key regulators of cell growth and development in response to nutrient availability. A discussion of the many functions of TOR kinases is not within the scope of this article and the reader is referred to De Virgilio & Loewith (2006) and Rohde et al. (2008) for recent, very accessible reviews. At least a portion of the TOR input into regulation of glycogen storage likely involves transcriptional control and there is a TOR component to the retention of the Msn2p/Msn4p proteins within the cytoplasm (Beck & Hall, 1999). When TOR activity is decreased in response to nutrient limitation or rapamycin treatment, uptake of Msn2p/Msn4p into the nucleus would be favored. However, TOR is also a key regulator of the process of autophagy (discussed below) and there is crosstalk between the TOR and Snf1p signaling pathways (Kamada et al., 2000; Rohde et al., 2008). Therefore, dissecting the precise role(s) for TOR kinases in the control of glycogen storage will likely prove difficult.

Regulation of GSY1 transcription

As mentioned above, the major isoform of glycogen synthase is encoded by the GSY2 gene (Farkas et al., 1991). The role of the minor isoform of the enzyme encoded by GSY1 has remained somewhat enigmatic. Promoter analysis of GSY1 by the LaPorte group provided evidence that the control of GSY1 expression was somewhat different from that of GSY2, implying that under certain conditions, GSY1 might make a more substantial contribution toward glycogen synthase activity (Unnikrishnan et al., 2003). The promoter of the GSY1 gene has been shown to contain STRE elements (Unnikrishnan et al., 2003). As with GSY2, a consensus Mig1p-binding site was also found, which confers regulation by the Snf1p-mediated glucose repression system (Unnikrishnan et al., 2003). Differences between the GSY1 and the GSY2 promoters emerged, however, because the GSY1 promoter also contained a binding site for Rox1p (Unnikrishnan et al., 2003). Rox1p functions as a transcriptional repressor of a variety of genes under aerobic conditions (Keng, 1992). The regulation of GSY1 expression by Rox1p, and the presence of Hap2p-binding sites in the GSY2 promoter, raises the possibility at least that the two isoforms of glycogen synthase could be regulated differentially by oxygen availability. Under aerobic conditions, the presence of heme would allow Rox1p production and inhibition of GSY1 expression. Conversely, the heme produced would allow the activation of Hap2/3/4/5 and enhancement of GSY2 expression. Under anaerobic conditions, the situation would be reversed. In addition to negative regulation by Rox1p, an additional negative regulatory element, referred to as N1, was identified in the GSY1 promoter (Unnikrishnan et al., 2003). Deletion of the N1 sequence was shown to enhance GSY1 promoter activity by as much as fivefold. Finally, there is evidence from a microarray study that Pho85p plays a role in the transcriptional repression of GSY1 (Carroll et al., 2001).

Control of glycogen synthase by phosphorylation and by glucose-6-P

Glycogen synthase is inhibited by reversible protein phosphorylation. Significant advances in our understanding of this control have emerged since the early work of Cabib and colleagues (Rothman-Denes & Cabib, 1970, 1971) and Francois & Hers (1988). In vivo labeling and limited proteolysis localized the phosphorylation sites of Gsy2p to the C-terminus of the protein (Hardy & Roach, 1993). The particular sites phosphorylated, Ser650, Ser654 and Thr667, were then identified using a site-directed mutagenesis approach (Hardy & Roach, 1993). Subsequent work has confirmed that all three sites can be phosphorylated in vivo (Gruhler et al., 2005).

Pho85p and the cyclins Pcl8p and Pcl10p phosphatase and inactivate glycogen synthase

A combination of classical biochemistry, genetic screening in yeast and molecular biology revealed that the cyclin-dependent protein kinase, Pho85p, was the catalytic subunit of a glycogen synthase kinase (Huang et al., 1996b; Timblin et al., 1996).

As discussed above, Pho85p is involved in a large variety of cellular processes, ranging from cell cycle progression to the control of acid phosphatase expression (Huang et al., 2007). The specificity of Pho85p kinase action arises through the association of the catalytic subunit with a variety of different cyclins (Pcls) that function as target subunits (Measday et al., 1997; Huang et al., 2007). The Pcls can be divided into two families based on the degree of similarity between their cyclin boxes. One family comprises Pcl1p, Pcl2p, Pcl5p, Pcl9p and Clg1p, and these Pcls are suggested to be involved in cell cycle controls. The other family, comprising Pcl6p, Pcl7p, Pcl8p, Pcl10p and Pho80p, is thought to be involved in metabolic controls (Measday et al., 1997).

An interaction between Pcl10p and Gsy2p was detected in two-hybrid studies, implying that Pcl10p might play a role in the phosphorylation of Gsy2p (Huang et al., 1998). Deletion of PCL10 had little impact on glycogen storage (Huang et al., 1998). However, the PCL10 gene is most
closer related in sequence to PCL8 and the deletion of both PCL8 and PCL10 resulted in an activation of glycogen synthase (increase in the $-+/+\text{glucose-6-P activity ratio}$) and an increase in glycogen storage (Huang et al., 1998). Therefore, Pho85p, in complex with either Pcl8p or Pcl10p, likely formed a glycogen synthase kinase. Formal proof of this hypothesis came from studies of tagged proteins expressed in yeast and from in vitro reconstitution experiments performed with purified, recombinant proteins (Huang et al., 1998; Wilson et al., 1999).

It has proven somewhat easier to generate stable recombinant Pcl10p than to produce recombinant Pcl8p (W.A. Wilson, unpublished data) and most work has therefore focused on this cyclin. Tagged Pcl10p, immunoprecipitated Wilson, unpublished data) and most work has therefore implied some role for this kinase in the control of glycogen storage. Overexpression of Pcl10p was also present in the incubation. The recombinant Pho85p–Pcl10p kinase complex phosphorylated Gsy2p to a stoichiometry of approximately 1.3 mol phosphate : mol protein, phosphorylation occurring preferentially at Thr667 and, to a lesser extent, at Ser654 (Wilson et al., 1999; Pederson et al., 2000). The Pho85p–Pcl10p kinase was incapable of phosphorylating Ser650 (Wilson et al., 1999).

As mentioned above, two-hybrid studies had hinted at a physical interaction between Pcl10p and Gsy2p. These results were confirmed using purified recombinant proteins, where it was established that Pcl10p bound directly to Gsy2p (Wilson et al., 1999). Further work in vivo using tagged proteins demonstrated that overexpression of Pcl10p could drive much of the cell’s pool of Pho85p into association with glycogen particles, presumably via this interaction with Gsy2p (Wilson et al., 1999). In effect, then, Pcl10p functioned as a targeting subunit that could direct the Pho85p catalytic subunit to the appropriate substrate within the cell. These observations were in agreement with previous work, showing that while Pho85p–Pcl10p complexes immunoprecipitated from yeast could phosphorylate Gsy2p effectively, they were poor kinases for another known Pho85p substrate, the transcription factor Pho4p (Huang et al., 1998). Conversely, immunoprecipitated Pho85p–Pho80p complexes were poor Gsy2p kinases, but highly effective at phosphorylating Pho4p (Huang et al., 1998).

Other kinases acting upon glycogen synthase

As mentioned previously, the sites Ser650, Ser654 and Thr667 on Gsy2p (and at least the sites corresponding to Ser650 and Ser654 in Gsy1p) are known to be phosphorylated in vivo (Gruhler et al., 2005). We have also shown that Pho85p–Pcl10p is capable of phosphorylating only Ser654 and Thr667 and cannot phosphorylate Ser650 (Wilson et al., 1999). What is the kinase that phosphorylates Ser650? In a genome-wide screen of deletion mutants with altered glycogen storage, it was determined that yak1 mutant cells had elevated glycogen (Wilson et al., 2002a). The YAK1 gene encodes a protein kinase of the DYRK (dual specificity, tyrosine phosphorylated and regulated kinase) family (Garrett & Broach, 1989; Werner-Washburne et al., 1991; Griffioen et al., 2001). The translocation of Yak1p to the nucleus appears to be TOR dependent and is also stimulated by rapamycin treatment (Schmelzle et al., 2004) and Yak1p functions in the signaling pathway that links TOR activity to the synthesis of ribosomal proteins (Martin et al., 2004). Yak1p also phosphorylates Pop2p, an RNase of the DEDD family (Moriya et al., 2001). Intriguingly, DYRK1A and YRK2, two mammalian DYRK family kinases that are related to Yak1p, are known to phosphorylate and inactivate the muscle isoform of glycogen synthase (Skurat & Dietrich, 2004). Phosphorylation occurs at the site referred to as 3α, which is clearly related in sequence to Ser650 in Gsy2p (Skurat & Dietrich, 2004). Considering the intimate links between Yak1p and nutrient-sensing systems, it might seem naive to assume that Yak1p would regulate glycogen storage through direct phosphorylation of Gsy2p. However, the similarities between Yak1p and the mammalian DYRK kinases did make this an intriguing hypothesis. Despite resulting in significant glycogen overaccumulation, the deletion of YAK1 did not cause activation of glycogen synthase, as determined by measurement of the $-+/+\text{glucose-6-P activity ratio}$ (Wilson et al., 2002a; W. Wilson, unpublished data). Therefore, it appears that Yak1p-mediated phosphorylation likely does not directly affect the catalytic activity of Gsy2p, although this must be tested by in vitro phosphorylation experiments.

The PAS kinase Psk2p has also been reported to phosphorylate glycogen synthase and psk2 deletion mutants show increased glycogen storage (Rutter et al., 2002; Wilson et al., 2002a). In this case, the phosphorylation has been shown to occur at Ser654 (Rutter et al., 2002). However, stoichiometric phosphorylation of Gsy2p using recombinant Pask2p did not alter the activity of glycogen synthase (W.A. Wilson & J. Rutter, unpublished data). As described above, control of glycogen storage via PAS kinases
likely occurs indirectly through the regulation of UDPG channeling.

**Phosphatases acting upon glycogen synthase**

There have been reports that mutations in several yeast protein phosphatase catalytic subunit genes affect glycogen accumulation (Peng et al., 1991; Posas et al., 1991, 1993; Clotet et al., 1995). However, it is now accepted that the type-1 protein phosphatase, encoded by GLC7, is the major phosphatase regulating glycogen storage (Feng et al., 1991). Glc7p carries out many different cellular functions through association with specific accessory proteins, referred to as targeting subunits (Hubbard & Cohen, 1993). GLC7 is an essential gene because there is a requirement for Glc7p function during mitosis (Hisamoto et al., 1995; Mackelvie et al., 1995). Certain alleles of glc7, most notably glc7-1, render cells glycogen-deficient (Cannon et al., 1994). Glycogen synthase is heavily phosphorylated and inactive in glc7-1 mutant cells. The mutation present in the glc7-1 allele prevents the interaction between Glc7p and a particular targeting subunit known as Gac1p. Gac1p is related in sequence to mammalian R_{Ca}, which functions to target the type-1 protein phosphatase catalytic subunit to glycogen particles in skeletal muscle (Stralfors et al., 1985; Tang et al., 1991; Francois et al., 1992; Stuart et al., 1994). Similarly, Gac1p directs Glc7p to glycogen particles in yeast (Stuart et al., 1994).

**Poorly defined inputs of the Snf1p and PKA pathways into post-translational control of glycogen synthase**

Both Snf1p and PKA have clear transcriptional inputs into glycogen storage. However, both proteins also appear to play roles in the post-translational regulation of glycogen synthase activity (Hardy et al., 1994; Francois & Parrou, 2001). As described above, Gsy2p expression is very low in cells where the PKA pathway is constitutively activated. However, the expression of GSY2 from a heterologous promoter in a bcy1 strain can bypass the transcriptional control, allowing a high level of Gsy2p production (Hardy et al., 1994). Glycogen accumulation still does not occur, though, because the Gsy2p is highly phosphorylated and inactive (Hardy et al., 1994). The mechanism by which PKA regulates Gsy2p phosphorylation remains unresolved. In terms of both the transcriptional and the post-translational control of glycogen synthase, there is a clear antagonism between the PKA and the Snf1p pathways. Regarding the post-translational regulation, the deletion of snf1 results in a similar phenotype to activation of the PKA pathway, which is hyperphosphorylation and inactivation of Gsy2p (Hardy et al., 1994). The Snf1p kinase is itself a phosphoprotein and phosphorylation at Thr210 is required for activation (Sutherland et al., 2003; Elbing et al., 2006; Hedbac & Carlson, 2008). The inactivation of Snf1p involves the removal of phosphate from Thr210 and is catalyzed by the Glc7p protein phosphatase in complex with the Reg1p targeting subunit (McCartney & Schmidt, 2001). The deletion of REG1 rendered Snf1p constitutively phosphorylated and active (McCartney & Schmidt, 2001). The deletion of REG1 also resulted in hyperaccumulation of glycogen, which could not be entirely accounted for by the modest increase in GSY2 expression resulting from the bypass of glucose repression (Huang et al., 1996a).

Additionally, Snf1p functions as part of a heterotrimer that contains Snf4p and one of three related proteins, collectively referred to as β-subunits, which are encoded by the SIP1, SIP2 and GAL83 genes (reviewed in Hedbac & Carlson, 2008). Intriguingly, Sip1p, Sip2p and Gal83p all contain a carbohydrate-binding sequence, which has been referred to as the glycogen-binding domain (Wiatrowski et al., 2004; Mangat et al., 2010). The Gal83p subunit has been shown to bind tightly to glycogen and could therefore provide a means of tethering the Snf1p kinase complex to glycogen particles (Wiatrowski et al., 2004). Mutation of residues implicated in glycogen particle binding resulted in increased glycogen storage as well as activation of transcription of a variety of genes, including GSY1 and GSY2 (Wiatrowski et al., 2004). Deletion of the entire glycogen-binding domain from Gal83p produced a constitutively active form of the Snf1p kinase (Mangat et al., 2010). The significance of the binding interaction between the Snf1p kinase complex and glycogen particles is unclear and, indeed, point mutations that influence glycogen binding of the Gal83p subunit have been shown to upregulate the transcription of a variety of Snf1p-regulated genes even in a yeast background that does not synthesize glycogen (Wiatrowski et al., 2004).

**The interplay between glucose-6-P and phosphorylation in the control of Gsy2p**

As mentioned above, post-translational regulation of glycogen synthase involves the interplay of two regulatory mechanisms, namely inhibition by reversible phosphorylation and activation by the allosteric modulator, glucose-6-P. Because the inhibitory effects of phosphorylation can be overcome by sufficient glucose-6-P, the activity state of glycogen synthase at any particular instance is a function of both the phosphorylation state of the enzyme and the glucose-6-P content of the cell. This interplay is well demonstrated by the observation that the glycogen-deficient phenotype of snf1 mutant cells, which arises in large part from hyperphosphorylation and inactivation of glycogen synthase, can be suppressed by mutations that elevate
glucose-6-P (Hardy et al., 1994; Huang et al., 1997). In addition to allosteric activation of glycogen synthase, glucose-6-P has been shown to both stimulate the dephosphorylation of glycogen synthase and to inhibit its phosphorylation (Francois & Hers, 1988; Huang et al., 1997). Whether these latter effects are mediated by binding to glycogen synthase or through regulation of kinases/phosphatases that act upon glycogen synthase remains unclear.

The availability of bacterially expressed, highly purified glycogen synthase and Pho85p–Pcl10p kinase complexes allowed a rigorous assessment of the properties of Gsy2p and an analysis of the precise effects of phosphorylation and glucose-6-P on the kinetic parameters of the enzyme (Pederson et al., 2000). Recombinant Gsy2p was found to have a −/+glucose-6-P activity ratio of approximately 0.4–0.5. Therefore, the enzyme, in its completely dephosphorylated state (as isolated from E. coli), retained some degree of glucose-6-P dependence (Pederson et al., 2000). Indeed, addition of glucose-6-P reduced the $K_m$ for both UDPG and glycogen and also increased the $V_{max}$.

Phosphorylation by the Pho85p–Pcl10p kinase complex to a stoichiometry of ~1.3 mol phosphate/mol Gsy2p induced a drastic change in the kinetic properties. The phosphorylated enzyme had a very low activity without glucose-6-P. Under these conditions, the $K_m$ for UDPG was considerably increased and, in fact, could not be determined because it was not possible to reach saturation. An increase in the $K_m$ for glycogen was also observed. When glucose-6-P was added, however, the kinetic properties of the phosphorylated enzyme were comparable to those of the non-phosphorylated enzyme in the presence of glucose-6-P. The ability of glucose-6-P to overcome the effects of phosphorylation allowed the development of a three-state model for the control of enzyme activity, which is illustrated in Fig. 4.

The crystal structure of any fungal or mammalian glycogen synthase is still not available and a complete understanding of regulation by phosphorylation and glucose-6-P must await the publication of such data. However, some progress has been made toward a better appreciation of how glucose-6-P and phosphorylation affect the kinetic properties of the enzyme since the publication of the last major review in this area. Reasoning that glucose-6-P would likely interact with glycogen synthase through binding to positively charged residues, systematic mutagenesis of Gsy2p was carried out, mutating clusters of basic residues to alanine. This approach led to the identification of two triple mutants (R579A/R580A/R582A and R586A/R588A/R591A) that had altered sensitivity to glucose-6-P and somewhat different properties (Pederson et al., 2000). Whereas the R586A/R588A/R591A mutant could be phosphorylated and inactivated similar to the WT enzyme by Pho85p–Pcl10p, this inactivation was not reversed by glucose-6-P addition.

The activity of the R579A/R580A/R582A mutant, on the other hand, was reduced relative to the WT enzyme in the absence of phosphorylation. Phosphorylation did still reduce the activity of this mutant, but only slightly. As with the R586A/R588A/R591A mutant, glucose-6-P was unable to restore activity to the phosphorylated enzyme (Pederson et al., 2000). Therefore, the R586A/R588A/R591A mutant was likely deficient in glucose-6-P binding, whereas the R579A/R580A/R582A mutant was probably defective in the transition between different activity states and was unable to reach the highest activity state (Fig. 4). These in vitro studies were extended by work in yeast and it was shown that normal glycogen accumulation required the activation of glycogen synthase by glucose-6-P (Pederson et al., 2004).

**Regulation of glycogen phosphorylase by phosphorylation**

Gph1p is activated by phosphorylation of a specific threonine residue, Thr31, and mutant Gph1p, where this threonine is converted to alanine and is essentially inactive (Lin et al., 1995). The crystal structure of yeast glycogen phosphorylase was resolved in both the phosphorylated and the dephosphorylated forms and an excellent mechanistic understanding of how phosphorylation at Thr31 serves to regulate activity is available (Lin et al., 1996, 1997). In vitro, Thr31 can be phosphorylated by PKA, activating the enzyme. However, despite considerable effort and several reports of partial purification, the physiological yeast glycogen phosphorylase kinase has not been identified (Becker et al., 1983; Pohlig et al., 1983). There has been more progress in the identification of Gph1p phosphatases and a
regulatory network that controls the dephosphorylation and inactivation of Gph1p is beginning to be unraveled (Tung et al., 1995; Zhang et al., 1995; Nigavekar et al., 2002; Tan et al., 2003; Wilson et al., 2005). At the center of this network lies Pho85p once again.

**Pho85p in the control of the phosphorylation state of glycogen phosphorylase**

The connection between glycogen phosphorylase and Pho85p was first established through studies of respiratory mutants and cells lacking functional SNF1 (Wang et al., 2001b; Wilson et al., 2002b, 2005). Respiratory mutants begin to synthesize glycogen as nutrients in the medium are depleted, just as WT cells do (Chester, 1968; Enjalbert et al., 2000; Wilson et al., 2002b). However, respiratory mutants obviously cannot survive on nonfermentable carbon sources, such as ethanol. When glucose is depleted from the growth medium, such cells are forced to consume their glycogen stores. In contrast, WT cells are able to maintain significant glycogen stores as they transition to oxidative metabolism. Respiratory mutants therefore appear to be glycogen-deficient relative to WT cells (Chester, 1968; Enjalbert et al., 2000; Wilson et al., 2002b). The glycogen storage defect in respiratory mutants is thus due to enhanced glycogen utilization rather than a defect in glycogen synthesis (Enjalbert et al., 2000). As such, one would not expect it to be suppressed by mutation of PHO85, which leads to dephosphorylation and activation of glycogen synthase. This was indeed the case and mutation of PHO85 in respiratory mutants yielded an initial increase in glycogen storage, but could not suppress the utilization of this glycogen as the cells ran out of fermentable sugar (Enjalbert et al., 2000; Wilson et al., 2002b). Indeed, glycogen degradation was actually enhanced in respiratory mutants that also lacked a functional PHO85 gene. This observation was explained by the finding that the deletion of PHO85 resulted in a substantial increase in glycogen phosphorylase activity, which was independent of PCL8 and PCL10 (Wilson et al., 2002b).

In vitro assays demonstrated that the ability to dephosphorylate and inactivate glycogen phosphorylase was substantially decreased in pho85 mutant cells (Wilson et al., 2005). Deletion of the related cyclins PCL6 and PCL7 resulted in a similar inability to dephosphorylate phosphorylase (Wilson et al., 2005). Therefore, Pho85p–Pcl6p or Pho85p–Pcl7p appeared to be required, either directly or indirectly, to activate a phosphorylase phosphatase. These data were consistent with the role for PCL6 and PCL7 in the control of glycogen phosphorylase that had been hinted at in earlier work (Wang et al., 2001b).

To identify the phosphorylase phosphatase, in vitro phosphorylase phosphatase assays were performed using yeast deletion mutants, each of which lacked either a protein phosphatase catalytic subunit or a protein phosphatase regulatory/targeting subunit (Wilson et al., 2005). Only strains in which either the SHP1 or GLC8 genes had been deleted showed any appreciable reduction in phosphorylase phosphatase activity (Wilson et al., 2005). Shp1p is a member of the ubiquitin regulatory X domain-containing protein family and is known to interact with Cdc48p, a component of the retrotranslocation machinery that moves ubiquitinated proteins from the endoplasmic reticulum into the cytosol (Schuberth et al., 2004). The precise link between Shp1p and Glc7p activity is therefore somewhat obscure, although the deletion of Shp1p had been reported previously to reduce Glc7p activity measured in cell extracts (Zhang et al., 1995).

Glc8p, on the other hand, is well characterized. Glc8p was known to interact physically with Glc7p (Ramaswamy et al., 1998; Ho et al., 2002). Indeed, Glc8p had been shown to function as a Glc7p activator and the deletion of GLC8 reduced Glc7p activity measured in cell extracts (Tung et al., 1995; Ramaswamy et al., 1998; Nigavekar et al., 2002). Glc8p was known to be a phosphoprotein and phosphorylation was known to be important for Glc8p function in vivo (Tung et al., 1995). In an elegant study that combined classical genetic techniques with the use of a library of yeast strains, each expressing one particular protein kinase fused to glutathione-S-transferase, Tan et al. (2003) clearly established that Pho85p functioned as the physiological Glc8p kinase. Furthermore, these workers also demonstrated that the cyclins responsible for directing Pho85p toward phosphorylation of Glc8p were Pcl6p and Pcl7p, and that phosphorylation of Glc8p by Pho85p–Pcl7p (or Pho85p–Pcl6p) was required for the full activity of the Glc7p phosphatase (Tan et al., 2003).

Taking all of the above observations together, we arrive at a model whereby Pho85p, in conjunction with the cyclins Pcl6p and Pcl7p, functions as a positive regulator of the Glc7p phosphatase. This positive regulation occurs via phosphorylation of Glc8p by a kinase comprising Pho85p and Pcl7p (or Pcl6p). The Glc7p phosphatase then dephosphorylates and inactivates glycogen phosphorylase (Fig. 5).

**Spatial aspects of glycogen storage**

Glycogen synthesis and degradation have long been considered to be cytoplasmic processes. It is now apparent, however, that separable pools of glycogen exist within the yeast cell and that the vacuole has a key role to play in the long-term maintenance of glycogen stores (Wang et al., 2001a; Wilson et al., 2002a). In addition, the key enzymes of glycogen synthesis and degradation, glycogen synthase and glycogen phosphorylase, show alterations in their subcellular distribution dependent on the glycogen content,
Fig. 5. Control of glycogen phosphorylase activity by Pho85p and Glc7p. Pho85p and the cyclin Pcl6p (or Pcl7p) form a complex that phosphorylates Glc8p. This phosphorylation serves to activate the Glc7p–Glc8p phosphatase complex, which then dephosphorylates and inactivates glycogen phosphorylase. Shp1p appears to be a positive regulator of bulk Glc7p activity, which acts independent of Pho85p, and is not specific to the regulation of phosphorylase phosphatase activity.

glycogen synthase entering the nucleus under certain conditions (W.A. Wilson, unpublished data). Therefore, neither glycogen itself nor the enzymes of its metabolism can be considered purely cytoplasmic. The experiments that led to these conclusions and the significance of the findings to studies of glycogen storage are discussed below.

Vacuolar glycogen stores and autophagy

The first indication that the vacuole might play a role in glycogen metabolism came from the application of a classical genetic screen (Wang et al., 2001a). Although the deletion of the PHO85 gene restored glycogen accumulation to a strain deleted for the SNF1 gene, deletion of the two cyclins known to target Pho85p toward glycogen synthase, Pcl8p and Pcl10p, apparently failed to do so and an snf1 pcl8 pcl10 triple mutant appeared to be glycogen deficient (Huang et al., 1998). A screen for multicopy suppressors of the glycogen storage defect of this triple mutant yielded six genes (Wang et al., 2001a). The gene recovered most frequently was ATG1, which encodes a protein kinase required for the process of autophagy (Matsuura et al., 1997).

Similar to glycogen accumulation, autophagy is induced when yeasts are exposed to nutrient-limited conditions. Starvation signals are sensed and passed to a complex of several proteins referred to as the preautophagosomal structure. The preautophagosomal structure then initiates the formation of a double-membrane structure, referred to as the isolation membrane, which expands and envelopes a region of the cytoplasm. Material within this region becomes incorporated into a double-membrane-bound vesicle known as an autophagosome as the leading edges of the isolation membrane fuse together. The autophagosome in turn fuses with the vacuole, releasing a single-membrane-bound autophagic body into the vacuole lumen. The membrane and contents of the autophagic body are then degraded within the vacuole (reviewed in Xie & Klionsky, 2007; Cebollero & Reggiori, 2009). Overexpression of ATG1 in a WT yeast background led to a modest increase in glycogen (and increased autophagy) (Wang et al., 2001a). Conversely, the deletion of ATG1 in an otherwise WT background resulted in reduced glycogen storage that became evident only after several days of growth (Wang et al., 2001a). In addition, it was determined that the deletion of SNF1 inhibited autophagy, whereas the deletion of PHO85 enhanced the process (Wang et al., 2001a). Therefore, a link between autophagy and glycogen storage capacity had been uncovered, with the ability to carry out autophagy being required for normal glycogen storage.

Careful analysis of the snf1 pcl8 pcl10 mutant strain revealed that it was capable of glycogen synthesis at the approach to the stationary phase. However, this glycogen was rapidly degraded and thus the strain appeared to be glycogen-deficient later in growth (Wang et al., 2001a). In addition to the effects on glycogen synthase regulation (suppressed by the deletion of both PCL8 and PCL10), the deletion of the SNF1 gene also inhibited autophagy (Wang et al., 2001a). Enhancement of autophagy by overexpression of ATG1 somehow restored glycogen storage. Potentially, the ability of PHO85 deletion to restore essentially normal glycogen accumulation and maintenance of stores to an snf1 mutant could be partly explained by the enhancement of autophagy observed in this strain as well as the effect on glycogen synthase regulation.

Why would enhancement of autophagy promote retention of glycogen stores? In WT yeast, the synthesis of glycogen begins in the late logarithmic phase and, during the stationary phase, glycogen undergoes partial consumption, between 24 and 48 h, presumably in correspondence with the metabolic reprogramming necessitated by the depletion of glucose and limitation of other nutrients (Francois & Parrou, 2001; Wang et al., 2001a). During this period, glycogen phosphorylase is active and it is at this time that autophagy mutants rapidly deplete their glycogen. In WT strains, there is then a phase of resynthesis and maintenance of glycogen stores up to 5–6 days, whereas autophagy mutants never re-establish high glycogen levels (Wang et al., 2001a). A model has been proposed in which there are two glycogen pools: cytosolic and vacuolar (Fig. 6). Defective autophagy means that no glycogen is delivered to the vacuole and also that no metabolic intermediates are exported from the vacuole. Both could reduce glycogen...
levels: in the absence of normally recycled intermediates, glycogen may be depleted and there would be no vacuolar glycogen store, protected from cytosolic phosphorylase. This vacuolar glycogen store would normally be accessed later in starvation through the activity of the vacuolar $\alpha$-glucosidase encoded by $SGA1$ (Yamashita & Fukui, 1985; Pugh et al., 1989; Wang et al., 2001a). Despite its description as a sporulation-specific gene, $SGA1$ does play a role in the glycogen metabolism of haploid cells during vegetative growth. This was demonstrated by gene deletion studies in which double mutants lacking both the $GPH1$ and the $SGA1$ genes were constructed. Deletion of $GPH1$ resulted in an overaccumulation of glycogen that was most apparent after prolonged growth (Hwang et al., 1989). Deletion of $SGA1$, on the other hand, did not result in an increase in glycogen storage, but did confer inhibition of glycogen degradation very late in the stationary phase (Wang et al., 2001a). Deletion of both $GPH1$ and $SGA1$ generated a strain that both overaccumulated glycogen in the stationary phase and maintained that glycogen later in growth (Wang et al., 2001a).

**Vacuolar acidification and glycogen stores**

Further evidence for the role of the vacuole in glycogen storage came from a genome-wide screen for deletion mutants that were either defective in glycogen storage or hyperaccumulated this compound (Wilson et al., 2002a). Of the 566 strains identified that had a glycogen content different from the WT, approximately 10% had functions related to vesicle trafficking or vacuole function. Of particular note, mutants in nine genes encoding structural components of the vacuolar proton-translocating ATPase (V-ATPase) and five genes encoding proteins required for V-ATPase assembly were recovered in the screen. The V-ATPase is a multiprotein complex that is required for the acidification of internal organelles, including the vacuole (reviewed by Kane, 2006). The V-ATPase genes identified...
represent an obvious functional cluster, making a robust link between the ability to acidify the vacuole appropriately and glycogen storage. Of the 14 deletion mutants linked to V-ATPase function that were identified as impacting glycogen storage, all except for two resulted in increased glycogen stores. This indicated that a defective V-ATPase resulted in stabilization of the vacuolar glycogen pool. In support of this proposal, a functional pathway of autophagy was found to be required for overaccumulation of glycogen in a vma10 mutant strain. When autophagy was blocked in the vma10 mutant strain by deletion of ATG1, glycogen overaccumulation was also blocked. Presumably, without its ATPase, the vacuole could not acidify and function normally. In particular, the degradative enzymes of the vacuole would not be fully active. As mentioned above, the glycoamylylase encoded by SGAl resides in the vacuole and, presumably, its activity would be impaired (Colonna & Magee, 1978; Yamashita & Fukui, 1985; Pugh et al., 1989).

The subcellular localization of glycogen synthase and glycogen phosphorylase

Over the past 15 years or so, evidence from mammalian systems has been steadily accumulating, demonstrating that the subcellular distribution of glycogen synthase varies under different conditions (Fernandez-Novell et al., 1992a, b, 1996, 1997; Ferrer et al., 1997; Garcia-Rocha et al., 2001; Cid et al., 2005; Ou et al., 2005; Prats et al., 2005, 2009). The subcellular distribution of yeast glycogen synthase had not been addressed in any detail, being limited to data generated in a high-throughput study (Huh et al., 2003). Recently, one of us (W.A.W.), addressed the localization of glycogen synthase within yeast cells through the expression of fluorescently tagged Gsy2p. It was determined that the subcellular distribution of glycogen synthase varied as a function of the glycogen content (W.A. Wilson, unpublished data). In cells that contained high levels of glycogen, Gsy2p was distributed throughout the cytoplasm. When glycogen was absent, the glycogen synthase and phosphorylase would lose this cytoplasmic anchor. In the case of glycogen synthase, a fraction of the protein then enters the nucleus.

What might Gsy2p do within the nucleus? Glycogen serves as a store of both carbon and energy. The freeing of glycogen synthase from its cytoplasmic tether to the glycogen particle as these stores reduced would therefore be a signal that carbon and energy reserves were low. The uptake of glycogen synthase into the nucleus might therefore represent a form of molecular ‘fuel gauge.’ It is possible that glycogen synthase could regulate transcription in response to energy availability by some as yet undetermined means.

Future perspectives

Glycogen storage by yeast was first described over a century ago and has been the subject of quite intensive study ever since. Despite decades of effort, and considerable progress, many questions still remain. We have made the best headway in understanding the transcriptional and post-translational regulation of the enzymes of glycogen storage. However, we must complete the characterization of the transcription factors involved in the regulation of both glycogen synthase and glycogen phosphorylase. Additionally, we still have to identify the protein kinase(s) responsible for the phosphorylation and activation of glycogen phosphorylase, as well as the additional kinase(s) acting upon glycogen synthase. Studies of the Gsy1p isoform of glycogen synthase are also still in their infancy and the potential differences in the regulation between this isoform and the major Gsy2p isoform are worthy of further investigation.

Our new appreciation of the role of the vacuole in glycogen storage opens up additional areas of study because it is now apparent that there are two, spatially distinct, pools of glycogen within yeast cells. Major questions arising from this observation include determining the size of the vacuolar pool in relation to the total cellular glycogen content, and ascertaining whether this pool is regulated differently from the overall glycogen levels that have traditionally been analyzed. In addition to consideration of the subcellular distribution of glycogen pools, the subcellular distribution...
Regulation of glycogen metabolism in bacteria and yeast

The regulation of bacterial glycogen metabolism

Enzymology and spatial aspects of the process

The enzymology of the glycogen biosynthetic and degradative processes is highly conserved in most bacterial species (Ballicora et al., 2003; Preiss, 2009). Figure 7 schematically represents the pathway of extracellular carbohydrate–glycogen conversion in Escherichia coli. Extracellular glucose is taken up and converted into glucose–6-P by the carbohydrate phosphotransferase system (PTS). Phosphoglucomutase (PGM) then converts glucose–6-P to glucose–1-phosphate, which, in the presence of Mg2⁺ and ATP, is converted into ADP-glucose (ADPG) and inorganic pyrophosphate by means of ADPG pyrophosphorylase (GlgC) (Ballicora et al., 2003). Using ADPG as the sugar donor nucleotide, bacterial glycogen is produced by the action of glycogen synthase (GlgA). The only known exception to this rule is Prevotella bryantii [a Gram-negative ruminal bacterium that lacks GlgC, and whose GlgA exclusively recognizes UDPG as a glucosyl donor (Lou et al., 1997)]. After chain elongation by GlgA, glycogen branching enzyme (GlgB) catalyzes the formation of branched oligosaccharide chains having α-1,6-glucosidic linkages. This occurs in two phases. First, GlgB cleaves a six to nine glucosyl units long oligosaccharide from a nonreducing end of a glycogen molecule and then transfers the cleaved oligosaccharide to a C-6 hydroxyl group of a glucose residue in another part of the glycogen molecule (Preiss, 2009). Unlike yeast and mammalian cells where glycogenin participates in the initiation of glycogen synthesis, no glycogenin analog has been described in bacteria, and several fully sequenced genomes of bacteria known to accumulate glycogen have failed to reveal the presence of glycogenin homologues. Moreover, Ugalde et al. (2003) provided strong evidence that bacterial GlgA can not only elongate α(1,4)-linked glucans, but can also form the primer required for the elongation process by catalyzing its own glucosylation.

Genetic evidence that PTS and PGM are involved in the conversion process from extracellular glucose to internal glycogen has been obtained with E. coli mutants deleted in either pts or pgm, both mutants displaying glycogenless phenotypes (Eydallin et al., 2007b; Montero et al., 2009 and references contained therein). Genetic evidence that GlgC is the sole enzyme catalyzing the production of ADPG has been obtained with glgC mutants such as the E. coli AC70R1-504 strain (Leung et al., 1986; Ballicora et al., 2003). This mutant displays an apparent glycogenless phenotype when exposed to iodine vapors. However, recent determinations of glycogen contents using more sensitive procedures have shown that AC70R1-504 cells, as well as other mutants totally lacking the GlgC function, can accumulate substantial amounts of glycogen (Eydallin et al., 2007a; Morán-Zorzano et al., 2007a). These findings add to the growing evidence of the occurrence of various important, but still unidentified sources of ADPG linked to glycogen biosynthesis in different bacterial species (Martin et al., 1997; Sambou et al., 2008). In this context, the identification of a trehalose glucosyltransferase (TreT) that catalyzes the reversible conversion of trehalose and ADP into ADPG and glucose in the archaeon Thermococcus litoralis is noteworthy (Qu et al., 2004). In any case, experimental work is still necessary to determine whether this novel enzymatic activity is actually involved in glycogen biosynthesis in this organism.

Glycogen metabolism is subjected to allosteric regulation of enzymes (Deutscher et al., 2006; Preiss, 2009). In general, GlgC activators in heterotrophic bacteria are key metabolites.
that represent signals of high carbon and energy contents within the cell, whereas inhibitors of this enzyme are intermediates of low metabolic energy levels. Exceptions to this rule are GlgCs from different Bacillus spp., as they are apparently unregulated enzymes (Ballicora et al., 2003). In the case of *E. coli*, fructose-1,6-bisphosphate activates GlgC, whereas AMP acts as an important inhibitor. In support of the important role of GlgC allosteric modulation in the regulatory aspects of glycogen metabolism, *E. coli* cells bearing a mutated GlgC form that is insensitive to AMP allosteric modulation (Govons et al., 1973), and purA cells impaired in the first committed step of AMP biosynthesis (Montero et al., 2009), both display glycogen-excess phenotypes. Allosteric regulation of GlgC has been extensively reviewed in recent works also including aspects relating to the structure–function relationships of GlgC, GlgA and GlgB (Ballicora et al., 2003; Preiss, 2009), to which readers are referred for further information.

Glycogen phosphorylase (GlgP) (which removes glucose units from the nonreducing ends of the glycogen molecule) and debranching enzyme (GlgX) participate in the degradation of glycogen during extended periods of substrate deprivation (Dauvillée et al., 2003; Alonso-Casajús et al., 2006) (Fig. 7). A strong and highly specific interaction between *E. coli* GlgP and the PTS component HPr was found by surface plasmon resonance ligand fishing (Deutscher et al., 2006). GlgP binding to HPr is maximal when HPr is totally phosphorylated. Furthermore, GlgP activity is increased when it binds to unphosphorylated HPr. Because the cellular concentration of HPr is much higher than that of GlgP, it has been proposed that GlgP activity is regulated by the phosphorylation status of Hpr, therefore allowing the accumulation of glycogen at the onset of the stationary phase under glucose-excess conditions (Deutscher et al., 2006).

Adenosine diphosphate sugar pyrophosphatase (AspP) catalyzes the hydrolytic breakdown of ADPG linked to glycogen biosynthesis (Moreno-Bruna et al., 2001). Its activity is positively affected by both glucose-1,6-bisphosphate and nucleotide–sugars and also by macromolecular crowding (Morán-Zorzano et al., 2007b). Increased macromolecular crowding can affect the activity, assembly status, complex formation and binding to the cell structures of many enzymes, and becomes more pronounced as cells enter the stationary phase (Makinoshima et al., 2003). This strongly suggests that AspP functions are also tightly regulated in the bacterial cell, likely playing a relevant role in preventing ADPG accumulation and in diverting the carbon flux from glycogen biosynthesis to other metabolic pathways in response to biochemical needs (Morán-Zorzano et al., 2008).

Glycogen granules and several enzymes involved in bacterial glycogen metabolism are localized in the cell periphery (Chambost et al., 1973; Pulkownik & Walker, 1976; Cattanéo et al., 1979; Spatafora et al., 1995; Makinoshima et al., 2003; Eydallin et al., 2007a; Morán-Zorzano et al., 2008) (Fig. 8), which suggests the possible occurrence of microcompartments, wherein the enzymes are physically associated in complexes (metabolons) to facilitate metabolite channeling. Thus, changes in macromolecular crowding and translocation of glycogen metabolism-associated enzymes in response to specific extra- and intracellular signals may regulate the formation of metabolic complexes and metabolite channeling, resulting in changes in the glycogen content (Morán-Zorzano et al., 2008).

**Organization of structural glycogen genes**

One of the salient features in bacterial gene expression is that genes of related functions are often clustered in a single operon, which ensures the simultaneous expression of functionally related gene products. A number of bacteria possess a single glycogen operon comprising all glg genes (Ugalde et al., 1998; Kiel et al., 1994; Marroqui et al., 2001; Lepek et al., 2002) (Fig. 9). However, it is widely accepted that genes involved in *E. coli* and *Salmonella* glycogen metabolism are clustered in two tandemly arranged operons: glgBX (encoding proteins that control the architectural aspects of the glycogen granule) and glgCAP (encoding proteins involved in the synthesis and breakdown of glycogen) (Preiss, 2009). The main evidence supporting this hypothesis comes from *in vitro* analyses showing that, unlike glgB, *E. coli* glgC and glgA expression is enhanced by the cAMP/cAMP receptor protein (CRP) complex and by guanosine 5’-(tri)diphosphate 3’-diphosphate (ppGpp) (Romeo & Preiss, 1989). These observations, however, conflict with recent transcriptomic analyses showing that the expression of the five glg genes (glgB, glgX, glgC, glgA and glgP) was not affected in different *E. coli* Δcrp mutants lacking CRP (Gosset et al., 2004; Zheng et al., 2004; Hollands et al., 2007) and also that all five *glg* transcript levels were downregulated to similar extents in *E. coli* ΔrelAΔspoT double mutants totally lacking (pp)Gpp production (Traxler et al., 2008). S1 nuclease protection assays identified up to four different transcripts initiating within a 0.5-kbp region upstream of *glgC* (Romeo & Preiss, 1989), indicating that (1) *glgCAP* promoter sequences are located in the region immediately upstream from *glgC* and (2) *glgBX* and *glgCAP* constitute two different operons. However, no *E. coli* consensus promoter sequences could be found in the region upstream from *glgC*, which comprises the 3’ end of *glgX* (Romeo & Preiss, 1989). In addition, Dauvillée et al. (2005) and Montero et al. (2009) have recently shown that mutants lacking the complete *glgX* gene display a glycogen-excess phenotype, indicating that significant levels of *glgCAP* transcription can also initiate upstream of *glgX*. Further
work is certainly needed to elucidate whether all *E. coli* glgBXCAP genes are significantly transcribed as a single mRNA unit, as well as the possible levels of regulation of the expression of these genes due to processing of this transcript.

**Regulation of the expression of glycogen genes**

Regulation of *E. coli* glycogen metabolism involves a complex assemblage of factors that are adjusted to the physiological and energetic status of the cell (Dietzler *et al.*, 1974; Eydallin *et al.*, 2007b; Montero *et al.*, 2009) and to cell-to-cell communication (Morán-Zorzano *et al.*, 2008). At the level of gene expression, several factors have been described to control bacterial glycogen accumulation. In *E. coli*, this includes negative regulation by the still unidentified glgQ regulatory locus, and by the carbon storage regulator CsrA (Romeo *et al.*, 1993; Yang *et al.*, 1996; Baker *et al.*, 2002), and positive regulation by RpoS, the PhoP/PhoQ regulatory system, the stringent response and by the cAMP/CRP complex.

RpoS is an alternative sigma factor of the RNA polymerase for the general stress response, which is required for normal glycogen biosynthesis (Lange & Hengge-Aronis, 1991; Eydallin *et al.*, 2007b, 2010; Montero *et al.*, 2009). *lacZ* fusion analyses on WT and rpoS mutant cells have shown that RpoS does not regulate glgCAP transcription in *E. coli* (Hengge-Aronis & Fischer, 1992; Eydallin *et al.*, 2010; Montero *et al.*, 2009), but positively controls the expression of glgS, a gene whose product exerts a positive, but still undefined effect on glycogen accumulation (Hengge-Aronis & Fischer, 1992; Eydallin *et al.*, 2010; Montero *et al.*, 2009). This gene codes for a 7.9-kDa protein, which is hydrophilic, highly charged and has no significant sequence similarity to any other
protein present in databases outside enterobacteria (Beglova et al., 1997; Kozlov et al., 2004). Sequence analyses of the 1000-bp-long promoter region upstream from the ATG initiation codon of glgS did not reveal the presence of a putative RpoS box, defined in E. coli as TGNCYATAMT (Lacour & Landini, 2004) or TCTATACCTAA (Weber et al., 2005). It is thus possible that glgS is indirectly regulated by RpoS, as has been shown to occur in many other genes belonging to the RpoS regulon (Weber et al., 2005). Intriguingly, however, recent transcriptome, proteome and lacZ fusion analyses failed to show that glgS belongs to the RpoS regulon (Lacour & Landini, 2004; Vijayakumar et al., 2004; Weber et al., 2005; Lelong et al., 2007).

During nutrient starvation, E. coli elicits the so-called 'stringent response' that switches the cell from a growth-related mode to maintenance/survival mode (Dennis et al., 2004; Potrykus & Cashel, 2008). The hallmark of this pleiotropic physiological response is the accumulation of the alarmones pppGpp and ppGpp (Potrykus & Cashel, 2008). While ppGpp is more abundant than pppGpp, the relative effects of these two regulatory nucleotides have not been examined thoroughly, their levels depending on the synthesis of pppGpp by RelA and SpoT, the hydrolysis of pppGpp to ppGpp by Gpp and the breakdown of ppGpp by the bifunctional enzyme SpoT (Hara & Sy, 1983; Xiao et al., 1991; Kuroda et al., 1997; Potrykus & Cashel, 2008). (p)ppGpp binds bacterial RNA polymerase to increase the transcription of amino acid biosynthesis genes during amino acid starvation and to downregulate the transcription of 'stable' RNAs (rRNAs and tRNAs) genes (Dennis et al., 2004; Potrykus & Cashel, 2008). As transcription of genes coding for components of the translation apparatus accounts for a large percentage of transcription in exponentially growing cells, the release of RNA polymerase from these genes is thought to passively allow upregulation of diverse promoters activated at the onset of the stationary phase (Barker et al., 2001). In this respect, different in vivo and in vitro experimental evidence has linked the E. coli stringent response and (p)ppGpp accumulation to increased glycogen content and enhanced expression of glg genes at the onset of the stationary phase (Bridger & Paranchych, 1978; Taguchi et al., 1980; Romeo et al., 1990; Traxler et al., 2008). Consistent with the involvement of (p)ppGpp in the regulatory aspects of glycogen metabolism, and also consistent with the assigned functions of SpoT and Gpp in (p)ppGpp degradation, Eydallin et al. (2007b) and Montero et al. (2009) have recently shown that E. coli cells impaired in the relA function display a glycogen-deficient phenotype as a consequence of the downregulation of glycogen gene expression. In addition, Eydallin et al. (2010) have shown that both spoT and gpp overexpressing cells display glycogen-deficient phenotypes. Data relating to the possible involvement of the stringent response in glgS expression are contradictory: whereas transcriptome analyses have recently shown that glgS is positively regulated by ppGpp (Traxler et al., 2008), similar types of analyses failed to detect glgS as a member of the RelA regulon (Durfee et al., 2008).

PhoP–PhoQ is a two-component regulatory system occurring in E. coli and Salmonella spp. that monitors the availability of extracellular Mg2+, transcriptionally controlling the expression of many genes (Garcia-Vescovi et al., 1996). Mg2+ is a stabilizing factor for membranes, tRNA, ribosomes, etc. that strongly determines cell metabolic and energetic status. In fact, changes in the external Mg2+...
concentrations in the submillimolar range have profound effects on the ability of *E. coli* to accumulate glycogen (Montero et al., 2009). Furthermore, *phoP* and *phoQ* mutants display glycogen-deficient phenotypes when cultured under conditions of limiting Mg$^{2+}$ concentration (Montero et al., 2009). Analyses of β-galactosidase activity levels derived from *glgC::lacZ* transcriptional fusions have shown that, under conditions of limiting Mg$^{2+}$ concentration, *glgC* expression is significantly lower in cells lacking the *phoP* and *phoQ* functions than in WT cells (Montero et al., 2009). Moreover, supplementation of the culture medium with Mg$^{2+}$ largely restores *glgC::lacZ* expression in *phoP* and *phoQ* mutants. In addition, Western blot analyses of GlgC revealed that both *phoP* cells and *phoQ* mutants accumulated lower levels of this protein than WT cells when cultured in the presence of low Mg$^{2+}$, the overall data indicating that the expression of the *E. coli glg* genes is under control of the PhoP–PhoQ system under low environmental Mg$^{2+}$ conditions (Montero et al., 2009). Noteworthy, sequence analyses of the region upstream from the ATG initiation codon of *glgC* did not reveal the presence of a putative PhoP box, defined in *E. coli* as (T)G(T)TT(AA) or (T/G)GT(CT)T tandem direct repeats (Yamamoto et al., 2002; Minagawa et al., 2003). It is thus possible that the PhoP–PhoQ-mediated Mg$^{2+}$ regulation of *glgCAP* is indirect.

CRP is one of the best-known global regulatory proteins in *E. coli*. cAMP produced by the membrane-bound adenylate cyclase (the product of *cya*) has been proposed to be a sensory signal in global gene control that acts through CRP (Kolb et al., 1993). Variations in the level of cAMP/CRP complex in response to the presence of extracellular glucose contribute to the fine regulation of several operons. Different studies have shown that *cya* and *crp* mutants display marked glycogen-deficient phenotypes (Leckie et al., 1983; Montero et al., 2009), which indicates that cAMP complexed to CRP (1) is required for normal glycogen accumulation and (2) serves as an important regulator of the transcription of genes involved in glycogen metabolism. cAMP/CRP is required for the expression of *glgS* and PTS-related genes (Hengge-Aronis & Fischer, 1992; Gosset et al., 2004), which in turn are required for normal glycogen production. As to the possible regulation of glycogen structural genes by the *cya* and *crp* gene products, *in vitro* experiments have shown that cAMP/CRP positively regulates the expression of *E. coli glgC* and *glgA*, but not that of *glgB* (Urbanowski et al., 1983; Romeo & Preiss, 1989; Romeo et al., 1990). However, recent transcriptome analyses failed to indicate that *E. coli glg* genes belong to the CRP regulon (Gosset et al., 2004; Zheng et al., 2004). Furthermore, using a chromosomal *glgC::lacZ* fusion constructed on a *cya* mutant, Montero et al. (2009) have recently shown that the expression of the *glgCAP* operon is not affected by the lack of cAMP production. The overall data thus indicate that the control of *E. coli* glycogen metabolism by cAMP/CRP may be due, at least in part, to the positive effect of this cyclic nucleotide on the expression of PTS-related genes rather than a direct effect on the expression of glycogen genes.

**Glycogen metabolism is highly interconnected with a wide variety of cellular processes**

Using the Keio collection of gene-disrupted mutants of *E. coli* (Baba et al., 2006) and the ASKA gene expression library (Kitagawa et al., 2005), studies have been recently carried out aimed to uncover the mechanisms regulating glycogen metabolism and its connection with other biological processes in *E. coli* (Eydamlin et al., 2007b, 2010; Montero et al., 2009). These studies have revealed that bacterial glycogen metabolism is highly interconnected with a wide variety of cellular processes, being affected by proteins that can be placed within the following groups: (1) stringent response, (2) general stress response, (3) low extracellular Mg$^{2+}$ availability, (4) carbon sensing, transport and metabolism, (5) factors determining intercellular communication, aggregative and social behavior, (6) sulfur metabolism, (7) nitrogen metabolism, (8) iron metabolism, (9) end-turnover of tRNA, (10) envelope composition and integrity, (11) energy production and cellular redox status, (12) small RNAs (sRNAs)-binding proteins, (13) nucleotide metabolism and (14) osmotic stress (Supporting Information, Tables S1 and S2). Functional groups (1)–(4) have been discussed above. Therefore, in the following sections, we will discuss the possible link between glycogen metabolism and groups (5)–(14).

**Factors determining intercellular communication, aggregative and social behavior modes**

Morán-Zorzano et al. (2008) have recently shown that AspP binds to cell membranes as the bacterial population density increases, remaining membrane-associated as glycogen depletes during the stationary phase. This process is stimulated by small soluble molecule(s) occurring in cell-free spent supernatants of stationary cultures, thus providing a first set of evidence that glycogen metabolism may be subjected to regulation by cell-to-cell communication. In *E. coli*, swimming, swarming and adherence of cells to surfaces or to one another by biofilm formation are fundamental modes to communicate and to regulate metabolic processes co-ordinately. Communication, aggregative and social behavior modes are highly determined by environmental cues, and act as major determinants of the nutritional status of the cell, which, as discussed above, is a major determinant of glycogen accumulation. Noteworthy, gaining-of-function of GGDEF and EAL domain enzymes controlling the intracellular levels of cyclic di-guanosine monophosphate...
Nitrogen and sulfur metabolism

It is known that carbon metabolism is subjected to regulation by nitrogen availability, although the mechanisms involved are still obscure. PtsN is a member of the nitrogen-related PTS, which has been associated with balancing of nitrogen and carbon metabolism (Reitzer et al., 1992). Consistently, ptsN overexpressing E. coli cells are characterized by a marked glycogen-deficient phenotype when cultured in glucose Kornberg medium (Eydallin et al., 2010).

Yeast extract (the amino acid source of the Kornberg medium) is deficient in amino acids such as cysteine (Reitzer, 1996; Eydallin et al., 2007b). Mutants impaired in functions involved in cysteine biosynthesis display a glycogen-excess phenotype when they are cultured in Kornberg medium (Montero et al., 2009), which can be ascribed to the stringent response-mediated upregulation of glg genes due to the lack of cysteine provision (Eydallin et al., 2007b).

Confirming this view, these mutants display a normal glycogen content phenotype when Kornberg medium is supplemented with cysteine. Because cysteine constitutes the almost exclusive metabolic entrance of reduced sulfur into cell metabolism, it is likely that the glycogen-excess phenotype of mutants impaired in cysteine biosynthesis is the result of the stringent response elicited by both nitrogen (amino acid) and sulfur starvation.

Lon and the two-component Clp ATP-dependent proteases play a major role in the degradation of damaged polypeptides and in the recycling of amino acids in response to a nutritional downshift, which is a process involving a major portion of the maintenance energy requirement (Gottesman, 2003). Cells impaired in lon, clpP or clpA display a glycogen-excess phenotype, which is ascribed to (1) deviation of energy flux from protein degradation to glycogen biosynthesis and (2) elicitation of the stringent response due to the lack of internal amino acid provision (Eydallin et al., 2007b; Montero et al., 2009), which, as discussed above, leads to enhancement of the glycogen content.

Iron metabolism

Under aerobic conditions, E. coli utilizes high-affinity extracellular siderophores that solubilize and capture Fe(III) before transport and metabolism. Fur, a dominant sensor of iron availability, generally represses iron siderophore biosynthetic and transport genes such as fepB, fepD and fepG (McHugh et al., 2003). Under iron-limiting conditions, iron dissociates from Fur, and increased transcription of genes ensues. Iron limitation causes the SpoT-dependent stringent response (Vinella et al., 2005), which, as discussed above, leads to enhancement of the glycogen content. Consistently, fepB, fepD and fepG mutants display glycogen-excess phenotypes, whereas fur mutants display a glycogen-deficient phenotype (Montero et al., 2009).

End-turnover of tRNA

End-turnover of tRNA consists of the removal and read- dition of the 3′-terminal AMP residues to uncharged tRNA. RNase T (the product of rnt) is a nuclease highly specific for uncharged tRNA–C–C–A that releases AMP and tRNA–C–C, and that highly controls tRNA turnover in E. coli (Deutscher et al., 1985). rnt mutants impaired in tRNA turnover accumulate defective tRNA molecules and high levels of ppGpp (Deutscher et al., 1977), thus resulting in increased glycogen content (Montero et al., 2009).

Envelope composition and integrity

RpoE is an essential transcription initiation factor that governs the response to envelope stress and the expression of genes that are needed to heal envelope damage. The major point of regulation of RpoE is at the level of its interaction with the antisigma RseA factor (Alba & Gross, 2004). When E. coli is subjected to extracytoplasmic stresses, RseA degrades and RpoE activity is induced. Another major point of regulation of RpoE takes place at the post-transcriptional level, because it has been shown that Hfq interaction with rseA mRNA downregulates rseA expression (Ding et al., 2004). Noteworthy, cells impaired in RseA and Hfq functions accumulate low glycogen levels, suggesting that RpoE-mediated envelope stress response may to some extent negatively affect glycogen accumulation (Montero et al., 2009). Mutants of genes coding for proteins involved in the maintenance of the cell envelope integrity such as rfaE, galU, tolB, tolR, tolQ, pal and ponB, display glycogen-deficient phenotypes (Montero et al., 2009). All these mutants are likely to promote both envelope stress membrane deformation that causes inhibition of the electron transport chain,
energy production and formation of membrane potential necessary for nutrient import (Andersen & Koepepe, 2007). The rfaE mutant for instance lacks an enzyme required for E. coli lipopolysaccharide biosynthesis (Valvano et al., 2000). Mutants impaired in the GalU function lack the enzyme that catalyzes the synthesis of UDPG necessary for the synthesis of cell envelope components (Genevaux et al., 1999). Moreover, tolB, tolR, tolQ and pal mutants do not possess proteins of the Tol–Pal system essential in maintaining envelope integrity (Lloubès et al., 2001). In addition, ponB (mrcB) mutants lack a bifunctional membrane-bound enzyme catalyzing transglycosylation and transpeptidation reactions, which are essential in the late stages of peptidoglycan biosynthesis (Plà et al., 1990).

Energy production and redox status

ATP is a primary signal in regulating glycogen biosynthesis, and acts as substrate for the ADPG-producing reaction catalyzed by GlgC (Preiss, 2009). Consistently, mutations in components required for the proper functioning of the aerobic electron transport chain and ATP generation negatively affect glycogen accumulation (Eydallin et al., 2007b; Montero et al., 2009). Thus, ubiG and ubiH mutants (deficient in ubiquinone production), iscU, iscS, fidX and hscB cells impaired in the machinery for the assembly/maintenance of Fe–S clusters (components required for the proper functioning of the aerobic electron transport chain and ATP generation), and gshB and gor mutants (lacking the machinery necessary to produce and reduce glutathione) showed reduced glycogen levels.

sRNAs-binding proteins

During the last decade, noncoding sRNAs have been shown to be involved in gene regulation. In prokaryotes, many sRNAs are specifically expressed during adaptation to nutritional stress (Gottesman, 2004). To date, close to 100 different sRNAs have been identified in E. coli, many of them regulating target mRNAs at the post-transcriptional level via direct base pairing, and, as a consequence, modifying translation and/or message stability. The majority of these sRNAs require binding proteins such as Hfq and CsrA for proper function in gene regulation.

The global regulator CsrA is a RNA-binding protein controlling a wide variety of biological processes, such as glycogen synthesis, glycolysis, motility and biofilm formation. It prevents glycogen accumulation by both promoting glgCAP decay and preventing glgC translation (Liu et al., 1995; Baker et al., 2002). Furthermore, CsrA negatively affects the expression of glgS (Yang et al., 1996) and hfq (Baker et al., 2007; Dong & Schellhorn, 2009). CsrA activity is antagonized by the two CsrB- and CsrC-noncoding sRNAs (Liu et al., 1997; Weibacher et al., 2003; Dubey et al., 2005), which in turn are targeted by CsrD for RNase E degradation (Suzuki et al., 2006). Consistent with the negative effect of CsrA in glycogen accumulation, and consistent with the assigned role of CsrD as relieving CsrA function from CsrB and CsrC in E. coli, recent studies have shown that csrA and csrD overexpressing E. coli cells display a glycogen-deficient phenotype (Eydallin et al., 2010). Noteworthy, and unlike the situation in E. coli, CsrA does not apparently play a relevant role in the regulation of glycogen accumulation in Salmonella enterica (Lawhon et al., 2003).

Hfq is a chaperone that stabilizes many regulatory sRNAs and facilitates the base pairing between sRNAs and their target mRNAs (Brennan & Link, 2007). The cellular role of the main part of the Hfq-binding sRNAs is to control stress responses. Deletion of hfq has pleiotropic phenotypes, including slow growth, altered cell division, osmosensitivity, increased oxidation of carbon sources, deficiency in the σE-mediated general stress response, accumulation of outer membrane proteins and altered patterns of protein synthesis (Valentin-Hansen et al., 2004). Mutants impaired in the hfq function have altered levels of expression of master transcription factors, and of genes affecting glycogen accumulation such as those involved in glucose transport, cytochrome metabolism, nitrogen metabolism, purine metabolism and glycogen structural genes such as glgB, glgX, glgC and glgA (Guisbert et al., 2007; Sittka et al., 2008). Noteworthy, these mutants also display a marked glycogen-deficient phenotype (Eydallin et al., 2007b; Montero et al., 2009) (1), showing that Hfq is necessary for normal glycogen accumulation and (2) suggesting the involvement of sRNAs in the regulation of E. coli glycogen metabolism. In fact, Hfq binds RprA and DsrA [both activators of RpoS (Majdalani et al., 2001)] and OxyS [a RpoS repressor (Gottesman, 2004)]. Hfq also binds RyaA, which strongly downregulates the expression of the glucose-specific PTS transporter encoding gene ptsG (Vanderpool & Gottesman, 2004). Furthermore, Hfq binds RybB and MicA (Figueroa-Bossi et al., 2006; Thompson et al., 2007), both acting as negative regulators of RpoE, which, as discussed above, exerts a negative effect in glycogen accumulation. Noteworthy, Hfq also binds gshS mRNAs (Zhang et al., 2003), strongly suggesting that the stability and/or the translation potential of gshS mRNAs is affected by Hfq.

Proposal of an integrated model for the regulation of glycogen metabolism in E. coli

Figure 10 illustrates a suggested model of glycogen metabolism in E. coli wherein major determinants of glycogen accumulation include levels of intracellular Mg-bound ATP necessary for ADPG synthesis [determined by extracellular
Mg\(^{2+}\) concentrations, transport of Mg\(^{2+}\) across membranes, ATP synthesis and consumption and adenylate kinase (Adk) activity (Igamberdiev & Kleczkowski, 2003), levels of AMP (the main GlgC inhibitor and indicator of the low energetic status of the cell), levels of ppGpp (which accumulates in a RelA-, SpoT- and/or Gpp-dependent manner under conditions of limited provision of nutrients such as amino acids, sulfur, iron, etc.), factors determining intercellular communication, aggregative and social behavior modes (which in turn determine the nutritional status of the cell), levels of cAMP expression levels of the general stress regulator RpoS and of the global regulators CsrA and Hfq, levels of cAMP, availability of a carbon source, redox status of the cell and less well-defined systems sensing the cell energy status through the activity of the electron transport chain (ETC). sRNAs, represented by stem-loops, are likely involved in the regulation of functions strongly affecting glycogen accumulation through interaction with CsrA and Hfq. According to this model, under conditions of limited nutrient provision, a decreased demand in ATP-dependent protein and nucleic acid synthesis will take place, and excess ATP will be used for glycogen biosynthesis when a carbon source is present in the medium.
Research needs

Over the past 5–10 years, improvements have been achieved in understanding how bacterial glycogen is regulated at the molecular level and how it is connected with other biological processes. A substantial part of this advance has been due to the availability of systematic and comprehensive gene disruption (Keio) and expression (ASKA) collections. However, considerable important information is still missing before a comprehensive understanding of the entire process can be attained. With emerging new techniques, the next decade will undoubtedly give us a better understanding of features, which, at present, are not completely understood such as the involvement of sRNAs in the regulation of carbohydrate metabolism in general and of glycogen in particular. In the short term, the identification and characterization of sources, other than GlgC, of ADPG (or any other glucosyl donor) linked to glycogen biosynthesis constitutes one of the major challenging objectives in the field of bacterial glycogen metabolism. Because glycogen appears to play relevant roles in the survival of bacteria to sporadic periods of famine, and because glycogen metabolism is highly interconnected with multiple and important cellular processes, it is tempting to speculate that redundancy of ADPG sources could have been selected during bacterial evolution to ensure the production of glycogen.

The precise molecular function of GlgS is still poorly resolved. This protein is important in the glycogen biosynthetic process, because it stimulates the synthesis of this polyglucan when overexpressed in the cell (Hengge-Aronis & Fischer, 1992; Eydallin et al., 2010). Earlier work suggested that GlgS might be a site for primary sugar attachment during the glycogen initiation process (Beglova et al., 1997). However, this hypothesis has been weakened by the finding that Agrobacterium tumefaciens GlgA does not require additional proteins for glycogen priming (Ugalde et al., 2003). The GlgS structure, suggesting a role in protein–protein interactions, offers alternative possibilities for its function (Kozlov et al., 2004). The stimulation of glycogen synthesis by GlgS overproduction may be caused by GlgS-mediated interactions between proteins involved in glycogen biosynthesis. As discussed above, changes in translocation of glycogen enzymes in response to specific extra- and intracellular signals would regulate the formation of metabolic complexes and metabolite channeling, which in turn would regulate glycogen metabolism. Protein–protein interaction analyses of GlgS would help to test this hypothesis. Noteworthy, protein complexes facilitating functional interactions among starch/glycogen metabolic enzymes have been recently shown to occur in plants (Tetlow et al., 2008; Hennen-Bierwagen et al., 2009).

As discussed by Montero et al. (2009), monitoring nutritional status is essential in optimizing bacterial survival strategies, including glycogen accumulation and/or breakdown. cAMP, (p)ppGpp and c-di-GMP are among the most comprehensively studied nucleotide-based second messengers. Although both cAMP and (p)ppGpp have long been linked to regulatory aspects of glycogen metabolism, only recently evidences have been provided suggesting the possible occurrence of c-di-GMP-mediated mechanisms regulating glycogen accumulation (Eydallin et al., 2010). c-di-GMP is an alarmone that is used by most bacteria to orchestrate the switch between free-living and sedentary life styles, controlling numerous cellular functions such as the biosynthesis of adhesions and exopolysaccharide matrix components, long-term survival and response to environmental stresses, synthesis of secondary metabolites, regulated proteolysis and cell cycle progression, etc. (Schirmer & Jenal, 2009). More work is needed to investigate the possible involvement of c-di-GMP in glycogen metabolism regulation and connection with other biological processes.

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Authors’ contribution

W.A.W. and M.M. contributed equally as first authors to this work on the yeast and bacterial sections, respectively.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

Table S1. List of genes (and functions of their products) whose ectopic expression affects glycogen content (Eydallin et al., 2010).

Table S2. List of genes (and functions of their products) whose deletion affects glycogen content (Eydallin et al., 2007b; Montero et al., 2009).

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